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Introduced Helicidae Garden Snails in Australia: Morphological and Molecular Diagnostics, Species Distributions and Systematics

MARK J. BLACKET,[1]* MICHAEL SHEA,[2] LINDA SEMERARO,[1] AND MALLIK B. MALIPATIL[1, 3]

[1] Department of Economic Development, Jobs, Transport and Resources (DEDJTR), Biosciences Research (BR), AgriBio, Centre for AgriBioscience, Bundoora Victoria 3083, Australia

[2] Australian Museum Research Institute, Sydney New South Wales 2010, Australia

[3] La Trobe University, Bundoora Victoria 3086, Australia

* mark.blacket@ecodev.vic.gov.au · http://orcid.org/0000-0001-7864-5712

ABSTRACT. There is a large number of Helicidae land snails native to the Western Palearctic, many of which have become invasive species in other parts of the world. In the past, multiple helicid species were introduced to Australia where they can now be major agricultural and horticultural pests. Determining which species have become established is essential for effective biosecurity and pest management. Here we have shown that three helicid species currently occur in Australia: Brown (*Cornu aspersum* Müller), Green (*Cantareus apertus* Born) and White (*Theba pisana* Müller) Garden Snails. A fourth formerly present species, the Chocolate Banded snail (*Eobania vermiculata* Müller) appears currently to be locally extinct. All four of these species are known to be highly invasive worldwide. Our study assessed the effectiveness of employing DNA barcoding for identification of garden snails in Australia through characterising DNA sequences of the mitochondrial Cytochrome Oxidase I and nuclear ITS2 loci. We were able to distinguish all four species, as well as other commonly intercepted Helicidae species. DNA sequences and diagnostic images of the helicid garden snails currently found in Australia have been added to the Barcode of Life Database (BOLD), as project AMPH (Australian Mollusc Pests—Helicidae), to aid in the identification of intercepted specimens, morphologically ambiguous individuals, or small juvenile specimens. We also examined the diagnostic morphological characters (juvenile and adult) that can be used to identify these species (including an illustrated key), and summarize relevant systematic and nomenclatural changes. We also provide the first specimen records for Green Garden Snails in eastern Australia, where they were previously unknown and may become a serious plant pest.

KEYWORDS. Land snails; Helicidae; introduced pests Australia; morphology; DNA barcoding; diagnostics; biosecurity.

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* author for correspondence

Members of Helicidae are land snails (Pulmonata: Stylommatophora) native to the Western Palearctic: Europe, Central Asia, the Middle East and Northern Africa, with more than 400 species currently recognised (Schileyko, 2006). Many species have expanded their ranges and are now cosmopolitan (Altaba, 2000; Cowie *et al.*, 2009; Peltanová *et al.*, 2012), including a number of species (Fig. 1) that have successfully established in Australia (Smith, 1992; Stanisic *et al.*, 2010), outlined further below. Introduced Helicidae species (garden snails) are associated with human activity, gardens and agriculture (Smith, 1992; Baker, 2002; Stanisic *et al.*, 2010), and can survive hot dry Australian summers through becoming dormant (aestivating). The pest status of Helicidae species in Australia is considered very severe, as they eat living plant material and are capable of causing extensive agricultural and horticultural damage (Kershaw, 1991; DAFWA, 2007; Stanisic *et al.*, 2010). Verifying which Helicidae species are currently present in Australia is essential for biosecurity and effective pest management.

The Brown Garden Snail, *Cornu aspersum* (Müller, 1774) (Figs 1, 2), is a cosmopolitan pest that has been repeatedly transported around the world (Guiller *et al.*, 2012; Peltanová *et al.*, 2012). This species was introduced to Australia soon after European settlement, possibly as a food source or imported accidentally in plant and soil material, from northern Europe (Smith, 1992). They were deliberately introduced into Tasmania (Petterd, 1879, cited in Petterd & Hedley, 1909) and in South Australia to eradicate slugs (Pomeroy & Laws, 1967). The Brown Garden Snail now occurs in all Australian states in many non-arid areas of southern and eastern Australia (Smith, 1992; Sanderson & Sirgel, 2002). The generic name of this species was recently under review, with debate as to whether it should be *Helix, Cornu, Cryptomphalus,* or *Cantareus* (Cowie, 2011), this species is currently recognized as *Cornu aspersum* (Stanisic *et al.*, 2010; ICZN, 2015).

Green Garden Snails, *Cantareus apertus* (Born, 1778) (Figs 1, 2), are also invasive worldwide (Godan, 1983; Cowie *et al.*, 2009). In Australia, they were first detected in the southwest coastal area of Western Australia in the 1980's where they have maintained a restricted distribution localized near Perth through quarantine control (Smith, 1992; DAFWA, 2007), with interceptions on produce resulting in restricted movement of plant material (e.g., Medlen, 2016). Until recently, Green Garden Snails were unknown from eastern Australia. However, the present study documents the first records of Green Garden Snails from eastern Australia (see below).

Two other Helicidae species (Fig. 1), White Garden Snails (also known in Australia as White Italian Snails), *Theba pisana* (Müller, 1774) and Chocolate Banded Snails, *Eobania vermiculata* (Müller, 1774), have also been introduced to Australia (Stanisic *et al.*, 2010). Both species are highly invasive worldwide (Deisler *et al.*, 2001; Roth & Sadeghian, 2003; Ueshima *et al.*, 2004; Cowie *et al.*, 2009; Neiber *et al.*, 2011). In Australia, *T. pisana* is currently present in all southern states, where it is a major grain (Baker, 2002), pasture (Baker, 2002) and grapevine (Sanderson & Sirgel, 2002) pest, with the major damage caused being contamination of harvested produce. The final helicid species, *E. vermiculata,* became established near Sydney, but now appears to be locally extinct (Stanisic *et al.*,

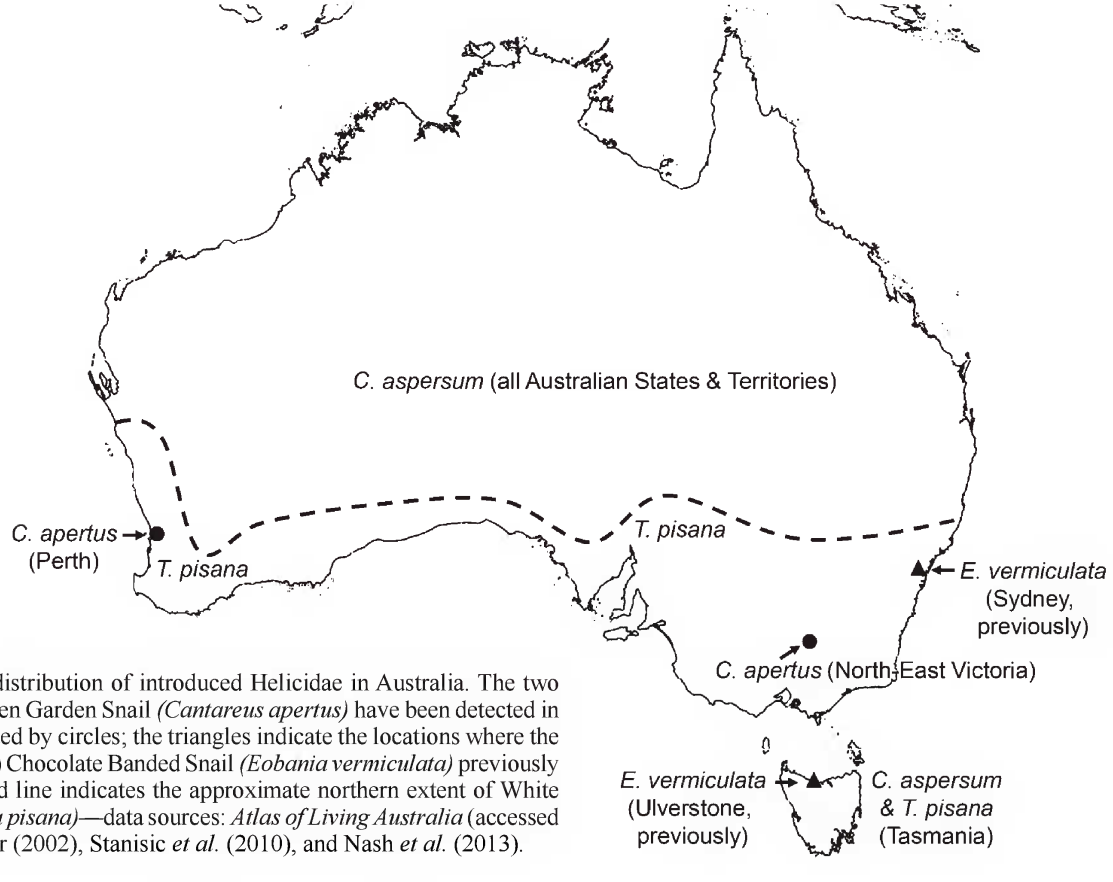

Figure 1. Current distribution of introduced Helicidae in Australia. The two locations where Green Garden Snail *(Cantareus apertus)* have been detected in Australia are indicated by circles; the triangles indicate the locations where the (now locally extinct) Chocolate Banded Snail *(Eobania vermiculata)* previously occurred; the dashed line indicates the approximate northern extent of White Garden Snail *(Theba pisana)*—data sources: *Atlas of Living Australia* (accessed March, 2015), Baker (2002), Stanisic *et al.* (2010), and Nash *et al.* (2013).

Figure 2. Images of live Brown *(C. aspersum)* and Green *(C. apertus)* Garden Snails. *(A)* Brown adult, Knoxfield Vic.; *(B)* Green adult, Cobram Vic.; *(C)* Brown adult, Knoxfield Vic. (left) and Green adult, Cobram Vic. (right); *(D)* Brown juvenile, Knoxfield (left ×1) and Cobram (right ×2) [note: these three specimen identifications (VAITC 3071–3073) were confirmed through DNA barcoding in the present study]; *(E)* Green Juvenile, Cobram Vic.; *(F)* Green epiphragm, Cobram Vic. Image credits: A–E, Andrew Henderson (DEDJTR); F, Gordon Berg (DEDJTR).

2010), with no specimens detected since the 1980's (Shea *et al., unpublished data*). Interestingly *E. vermiculata* has also been recorded as being abundant in northern Tasmania in the past (Petterd & Hedley, 1909), where it was formerly known from Leven River, Ulverstone Tasmania, but apparently no longer occurs there (K. Bonham, pers. comm.).

In addition to the four species outlined above, literature records suggest that a number of other Helicidae species may have established in Australia (Table 1). Smith (1992), listed the Milk Snail, *Otala lactea* (Müller), from Sydney and suggested that it is highly probable that Escargot, *Helix pomatia* (Linnaeus), might be present in Australia illegally for commercial purposes. Literature records suggest that Green Garden Snails have been found in Tasmania (Petterd & Hedley, 1909; Kershaw, 1991), however, this appears to be due to a nomenclatural error (see *Discussion*). Indeed, there have been a large number of recent nomenclatural changes, including recognition that many introduced snails previously regarded as Helicidae actually belong to Hygromiidae and Cochlicellidae (Table 1). Currently, seven species (in six genera) of Helicidae are listed as being "Recorded in Australia" based on museum records on the *Atlas of Living Australia* (ALA, http://www.ala.org.au/, accessed March 2015), including specimens of *Cepaea nemoralis* (Linnaeus), *Helix pomatia,* and *Otala lactea*, see Table 1. However it is, in many cases, unclear if these records represent specimens collected overseas housed in Australian collections, interceptions of exotic species at the nation's borders, or the establishment of particular species in Australia.

Morphological identification of Helicidae species can be difficult, with forty-four families of native terrestrial snail known from eastern Australia (Stanisic *et al.,* 2010), and an additional forty (Smith, 1992) to fifty (Sanderson & Sirgel, 2002) exotic species established in Australia. The large degree of morphological variation present in some snail species can make identification of morphologically similar species problematic, especially for juvenile specimens. Potentially DNA barcoding could aid in snail identification, however, it is essential that reference DNA sequences match vouchered specimens. Indeed, many Helicidae DNA sequences on GenBank have been shown to have been previously assigned to incorrect species (Groenberg *et al.,* 2011).

The three major aims of the present study were: *(a)* to review which Helicidae species have become established in Australia through an examination of literature and specimen records; *(b)* to examine diagnostic characters, both morphological (juvenile and adult) and molecular, to allow identification of Helicidae species in Australia; and *(c)* to provide a formal first record for Green Garden Snails from eastern Australia.

Materials and methods

Specimens examined

Juvenile and adult Australian specimens of four introduced Helicidae species: *Cornu aspersum*, *Cantareus apertus*, *Theba pisana*, and *Eobania vermiculata*, were examined morphologically (specimen details given in Table 2, and *Systematics* section). A series of Victorian specimens of *C. apertus* (from near Cobram), *C. aspersum* and *T. pisana* were examined for molecular variation (Table 2, n = 23), see below. The shells of these specimens were photographed with a Canon 5D digital SLR camera. Diagnostic images and associated DNA sequences are presented (this study) and have been submitted to the Barcode of Life Database (BOLD) as project AMPH: Australian Mollusc Pests—Helicidae. Specimens examined for molecular variation were prepared by freezing overnight, followed by removal of the body in 100% ethanol; specimens relaxed for morphological

examination of the soft body were prepared as in Stanisic *et al.* (2010), and preserved in 70% ethanol. Reference collection abbreviations: *AM*, Australian Museum; *VAIC*, Victorian Agricultural Insect Collection; *VAITC*, Victorian Agricultural Insect Tissue Collection (the VAIC and VAITC collections also include molluscs and other invertebrates).

Morphological variation

Six shell measurements were taken from Australian specimens of all four Helicidae species, from the axial-aperture view, as in Fig. 3 (specimens listed in Table 2, n = 89): *(1)* SW: shell width; *(2)* AW: aperture width, from the edge of columellar plait to edge of lip; *(3)* SH: shell height; *(4)* BW: height of body-whorl; *(5)* AH: aperture height, from the point of adhesion of aperture to edge of lip; *(6)* W: height of whorls (excluding the body-whorl), calculated from SH–BW measurements. All measurements, to the nearest 0.1 mm, were obtained using electronic digital callipers. Statistical tests, Discriminant Function Analyses and Principal Component Analyses, were performed using XLSTAT 2015 in Excel 2010 to assess differences between species and lifestages (note, in the present study there were no very small *E. vermiculata* available for examination). The general morphology of the shell and the soft body—the mantle, head and foot—were also examined for potential interspecific size, colour and pattern differences (in *E. vermiculata* only shells were available).

Molecular variation

DNA extractions were performed on approximately 3 mm^2 of foot tissue removed from ethanol preserved specimens with a single-use scalpel blade, using a commercially available kit (Dneasy® Blood and Tissue Kit, Qiagen), following the manufacturers protocol. Genetic variation was examined in two loci, the mitochondrial Cytochrome Oxidase I (COI) locus, and the nuclear Internal Transcribed 2 (ITS2) region. COI was amplified using primers LCO / HCO (Folmer *et al.,* 1999) as in Régnier *et al.* (2011), while novel ITS2 primers, Snail_ITS2-F (5'-GACATCTTGAACGCAMATGG-3') and Snail_ITS2-R (5'-TCACTCGCCGTTACTGRGG-3'), were designed using Primer 3 (Rozen & Skaletsky, 2000) to match Helicidae sequences on GenBank (from Wade et al., 2001, 2006), to amplify the ITS2 region, overlapping with data from Wade *et al.* (2001, 2006) and Colomba *et al.* (2011). PCR profiles for both loci involved an initial denaturation step of 94°C for 2 minutes, 40 amplification cycles of 30 second steps (at 94°C, 52°C and 72°C for COI, and 94°C, 55°C and 72°C for ITS2), and a final extension step of 72°C for 2 minutes. DNA sequencing was conducted commercially through Macrogen Inc. (Korea). The COI and ITS2 sequences obtained here have been submitted to GenBank (Table 2).

Blastn searches of both COI and ITS2 DNA sequences were conducted on NCBI GenBank to determine the closest species match for each DNA sequence. Additional estimates of COI variation and relationships between species / genera (using a Neighbour-joining tree, pairwise "p" sequence distances) were conducted in MEGA version 6 (Tamura *et al.,* 2013), including a sequence representative from many of the Helicidae genera currently available on GenBank (from Ansart *et al.* [unpublished *Helix, Cantareus*]; Däumer *et al.,* 2012; Dinapoli *et al.,* 2010; Elejalde *et al.,* 2008; Grande *et al.,* 2004; Greve *et al.,* 2010; Grindon & Davidson, 2013; Groenenberg *et al.,* 2011; Haase & Misof, 2009; Kotsakiozi *et al.,* 2012; Neiber *et al.,* 2011; Rada *et al.,* 2012; Régnier *et al.,* 2011). Phylogenetic analyses of the ITS2 sequences obtained in the present study were not conducted, due to the presence of a large number of alignment gaps between species and indels within species (see below).

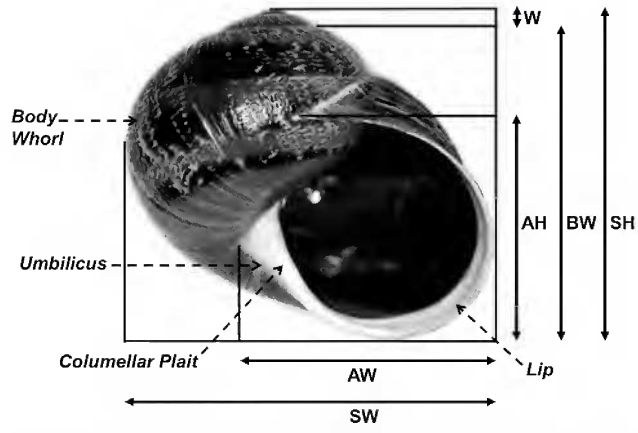

Figure 3. Snail shell with characters mentioned in the text labelled (dashed arrows). Locations of shell measurements are indicated by solid arrows and lines. *Abbreviations: AH:* aperture height; *AW:* aperture width; *BW:* body-whorl height; *SH:* shell height; *SW:* shell width; *W:* height of whorls, calculated from SH–BW.

Results

Helicidae species present in Australia

In the present study three Helicidae species were verified as being currently established in Australia: *C. aspersum*, *C. apertus*, *T. pisana*. A fourth species, *E. vermiculata,* became locally established twice, in NSW and Tasmania, but now appears locally extinct in both locations. No evidence for any additional species in Australia referred to in previous literature (see above and Table 1) were found. Indeed, reference collection specimens were not located in the present study to support previous literature records for *E. vermiculata* from northern Tasmania (Petterd & Hedley, 1909) or for *O. lactea* from NSW (Smith, 1992). It appears likely that previous NSW records of *O. lactea* were actually misidentified *E. vermiculata* (Shea, unpublished data) and that *E. vermiculata* was formerly known from northern Tasmania (K. Bonham, pers. comm.), but is not represented in reference collections. A number of other exotic Helicidae species are regularly intercepted at the nation's border (e.g., Table 3), with more than 900 interceptions occurring between 2002 and 2015, including at least 14 different Helicidae species (L. Watson, pers. comm.); however, none of these are known to have led to additional Helicidae species becoming established in Australia. Images, and adult shell sizes, of some exotic species commonly misidentified or intercepted at the border are shown in Fig. 4.

Morphological identification of Helicidae in Australia

Most native terrestrial snail species are small, subglobose, and not domestic in habit (Kershaw, 1991; Smith, 1992; Stanisic *et al.,* 2010). An up-to-date family key for eastern Australia is available to aid in identification (Stanisic *et al.,* 2010). A number of introduced species of Hygromiidae and Bradybaenidae are morphologically and ecologically similar to species of Helicidae (Stanisic *et al.,* 2010). Bradybaenidae is currently represented by a single species in Australia, the Asian Trampsnail *Bradybaena similaris* (illustrated in Stanisic *et al.,* 2010). Hygromiidae is a large Western Palearctic family with numerous species now established in Australia (e.g., Smith, 1992; Stanisic *et al.,* 2010). Hygromids vary widely in shell shape, but their shells do not usually possess a reflected lip or have a differentiation between the lip edge and the columella, the external soft body is similar in appearance to Helicidae but usually has a narrower foot (Stanisic *et al.,* 2010). Other more detailed descriptions of diagnostic Helicidae/Hygromiidae characters are outlined in Schileyko (2005, 2006), and a large number of potential exotic species are covered by Welter-Schultes (2012).

Some native snails, including many species of Camaenidae, which is one of the largest families in Australia and the dominant group in the tropics, could potentially be confused with Helicidae snails in being both globose and large in size (Stanisic *et al.* 2010). Species of Camaenidae differ from Helicidae/Hygromiidae in not possessing a dart sac and in having an eversible "head wart" (which emits an attractant pheromone) situated between the two superior tentacles (Stanisic *et al.,* 2010).

Specimens of the four Helicidae Garden Snail species collected from Australia, that are larger than c. 10 mm in diameter (specimens smaller than this often have fewer whorls and variable coloration), can be identified using the diagnostic images presented here (Figs 2, 5–7) and the following key:

Key to Helicidae species in Australia

1 Shell globose, with whorls increasing rapidly in width, 3–5 whorls, body-whorl greatly flared, aperture large and rounded, umbilicus closed, shell colour brown or green either with brown spiral bands or without a pattern (Figs 5–7, A and B) 2

—— Shell subglobose, with whorls increasing gradually in width, 5 whorls, body-whorl moderately flared, aperture small to medium, umbilicus narrow or closed, shell colour pale with brown spiral bands (Figs 5–7, C and D) 3

2 Shell large size (up to 4 cm in diameter, axial view), light brown colour with darker spiral bands and yellow flecks, raised spire, 4–5 whorls, aperture large relative to body. In adults spire more developed and lip of shell thickened white and strongly reflected out. During aestivation possesses either thin and clear or thickened and greyish green epiphragm that is not convex positioned inside aperture (Figs 2, 5–7, A) ..... *Cornu aspersum*

—— Shell medium size (up to 3 cm in diameter, axial view), olive-green (juveniles) to olive-brown (adults) colour with no banding pattern, low spire, 3–4 whorls, aperture extremely large relative to body. In adults lip of shell only thickened and white internally and not reflected out. During aestivation possesses distinctive convex white thick epiphragm (Fig. 2F) extending from edge of aperture (Figs 2, 5–7, B) ..... *Cantareus apertus*

3 Shell relatively depressed, medium size (up to 3 cm in diameter, axial view), light brown to yellow usually with continuous thick dark brown and white spiral bands and yellow flecks, rounded whorls, usually closed umbilicus, aperture small compressed shape. In adults lip of shell thickened white and strongly reflected out (Figs 5–7, C) ..... (locally extinct) *Eobania vermiculata*

—— Shell relatively tall, small size (up to 2 cm in diameter, axial view), white usually with broken thin dark brown spiral bands and chevrons, whorls angulate in juveniles rounded in adults, narrow umbilicus, aperture medium size rounded. In adults lip of shell internally thickened and pink not thickened externally or reflected out (Figs 5–7, D) ..... *Theba pisana*

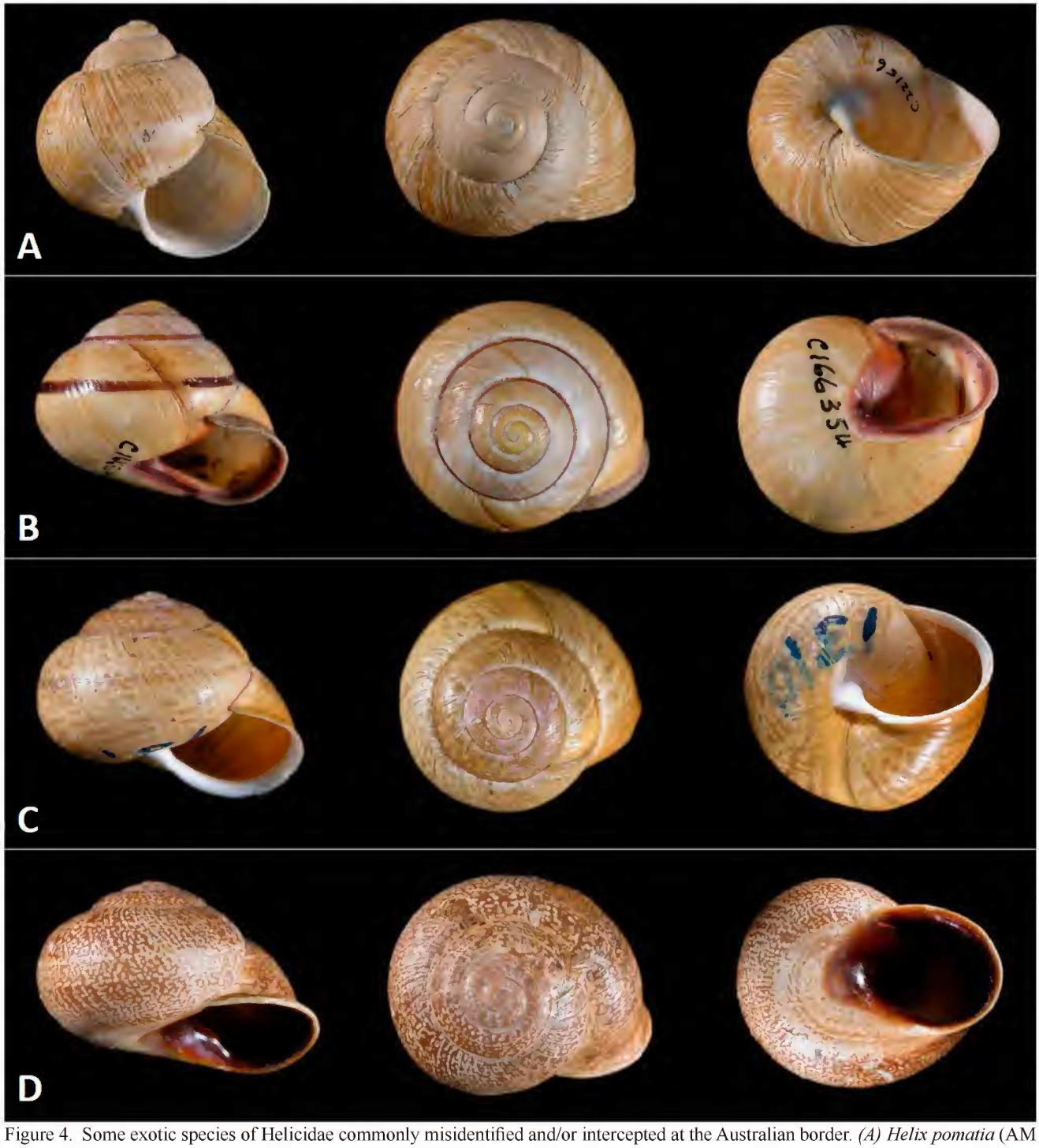

Figure 4. Some exotic species of Helicidae commonly misidentified and/or intercepted at the Australian border. *(A) Helix pomatia* (AM C22156), SW=30–50 mm; *(B) Cepaea nemoralis* (AM C166354), SW=18–25 mm; *(C) Arianta arbustrorum* (AM unregistered England), SW = 18–25 mm; *(D) Otala lactea* (AM unregistered, Portugal), SW = 30–40 mm. (Images are adults, not shown to same scale). Image credits: A–D, Des Beechey (AM).

Shell morphology

As well as the characters listed above, many shell size measurements appear different between the four species of garden snail (Table 4). Principal Component Analyses indicate that all variables contribute to differences between species (Table 4). However, most shell traits appear strongly correlated with each other (correlations >0.7, tolerance statistic <0.05), which is probably due to correlations of each trait with size (Table 4). Moderate correlations (0.49–0.52) were observed between trait W with AH and BW, reflecting between-species differences in shell shape (Fig. 8). Bivariate plots of shell measurements differ for each species (Fig. 8), with some relative proportions showing almost no overlap between species, or between conspecific adults and juveniles (Fig. 8). Discriminant Function Analyses of shell measurements (Table 4) indicated that most (>90%) specimens could be statistically assigned to the correct species and lifestage, based on confusion matrix estimates. Instances of incorrect assignment of specimens involved a number of juvenile *C. aspersum* (×2) and *C. apertus* (×2) specimens assigned as juveniles of the other species, and assignment

Table 1. Taxa that were regarded as Helicidae in selected past Australian field guides and checklists. Grey shading indicates that a species was not included in that particular geographically-localised reference, dashes indicate that species was included but not as Helicidae. Geographic regions in square brackets.

	Smith & Kershaw (1979) [S.E. Australia]	Kershaw (1991) [Tasmania]	Smith (1992) [Australia]	Stanisic *et al.* (2010) [Eastern Australia]	ALA[c] (2015) [Australia]	present in Australia	snail family (2015)
Brown Garden Snail	*Helix (Crypto-mphalus) aspersa*	*Helix (Crypto-mphalus) aspersa*	*Helix (Cornu) aspersa*	*Cornu aspersum*	*Cantareus aspersa* (Müller, 1774)	yes	Helicidae
Green Garden Snail		*Cantareus aperta*	*Helix (Cantareus) aperta*		*Cantareus apertus* (Born, 1778)	yes	Helicidae
Chocolate Banded Snail	*Eobania vermiculata*		*Eobania vermiculata*	*Eobania vermiculata*	*Eobania vermiculata* (Müller, 1774)	formerly	Helicidae
White Garden Snail	*Theba pisana*	*Theba pisana*	*Theba pisana*	*Theba pisana*	*Theba pisana* (Müller, 1774)	yes	Helicidae
Milk Snail			*Otala (Otala) lactea*		*Otala (Otala) lactea* [a] (Müller, 1774)	no [a]	Helicidae
Pointed Snail	*Cochlicella acuta*		*Cochlicella acuta*		*Cochlicella acuta* (Müller, 1774)	yes	Cochlicellidae
Small Pointed Snail	*Cochlicella ventrosa*	*Cochlicella barbara*	*Cochlicella barbara*	—	*Prietocella barbara* (Linnaeus, 1758)	yes	Cochlicellidae
Vineyard Snail	*Cernuella (Cernuella) virgata*		*Cernuella (Cernuella) virgata*	—	*Cernuella (Cern.) virgata* (Da Costa, 1778)	yes	Hygromiidae
Citrus Snail [b]	*Cernuella (Micro-xeromagna) vestita*	*Cernuella (Micro-xeromagna) vestita*	*Cernuella (Micro-xeromagna) vestita*	—	*Microxeromagna lowei* (Potiez & Michaud, 1838)	yes	Hygromiidae
Dune Snail	*Cernuella (Xerocinta) neglecta*	*Cernuella neglecta*	*Cernuella (Xerocinta) neglecta*	—	*Xerocincta neglecta* (Draparnaud, 1805)	yes	Hygromiidae
Wrinkled Dune Snail	*Candidula intersecta*		*Candidula intersecta*		*Candidula intersecta* (Poiret, 1801)	yes	Hygromiidae

a Note: *Otala lactea, Helix pomatia* and *Cepaea nemoralis* are all currently listed on the *ALA* (accessed March 2015) as "Recorded in Australia". However, these appear to refer to intercepted specimens, with these species not currently established in Australia.

b Note: Citrus snail is missing from the ALA (accessed March 2015), but is currently recognized as *Microxeromagna lowei* (Potiez & Michaud, 1838).

c ALA = Atlas of Living Australia · http://www.ala.org.au

Table 2. Number of adult and juvenile specimens examined for morphological measurements, molecular variation and DNA barcoding. Asterisks indicate collection localities for specimens photographed in Figs 2, 5–7.

species	location	adults	juveniles	GenBank accession no. (COI)	GenBank accession no. (ITS2)
Cornu aspersum	Doncaster, Vic	2	1 *	KX679317	KX679294
	Irymple, Vic	3 *	8 *	KX679314, KX679322	KX679292, KX679299
	Knoxfield, Vic	—	7	KX679320	KX679297
	Preston, Vic	—	4	KX679321	KX679298
	Ringwood East, Vic	1 *	4	KX679319	KX679296
	Blackburn South, Vic	—	1 *	KX679318	KX679295
	Cobram, Vic	—	2 *	KX679315, KX679316	KX679293
Cantareus apertus	Neerabup, WA	—	2	N/A	N/A
	Cobram, Vic	2 *	8 *	KX679306, KX679307, KX679308, KX679311	KX679284, KX679285, KX679286, KX679289
	Cobram South, Vic	—	9 *	KX679309, KX679310, KX679312, KX679313	KX679287, KX679288, KX679290, KX679291
Eobania vermiculata	Bronte, NSW	18 *	—	N/A	N/A
	Ryde, NSW	—	4 *	N/A	N/A
	Ryde, NSW	—	4 *	N/A	N/A
Theba pisana	3 km N Port Campbell, Vic	3	—	N/A	N/A
	Port Campbell, Vic	3	—	N/A	N/A
	Venus Bay, Vic	5 *	5 *	KX679323 – KX679328	KX679300 – KX679305

of one juvenile *E. vermiculata* as an adult *T. pisana*, with an additional four specimens within these latter two species assigned to the incorrect lifestage. In all species juvenile specimens appear generally paler in colour, possess a thinner shell-lip and have relatively lower spires than adults (Figs 2, 5–8). The degree of development and angle of the columellar plait also differs between species (Fig. 5), particularly in adult *E. vermiculata* where it occurs at a very shallow angle.

The colour and pattern of garden snail shells is extremely variable, and may be related to geographic and/or ecological factors (Johnson, 1980). Baker (2002), has previously reported that the shells of South Australian *T. pisana* are commonly entirely white. Indeed, it has been well documented that some helicid species that are normally patterned can have non-banded forms, e.g., *T. pisana*—Deisler *et al.,* 2001 and *E. vermiculata*—Yildirim, 2004. In the present study specimens examined were patterned as described in the above key.

Of the four species examined Brown and Green Garden Snails appear most similar and can be very difficult to identify as juveniles (Fig. 2). Indeed, in the present study reliable identifications of very small specimens of these two species were only possible through DNA barcoding (e.g., Fig. 2D). In addition to being non-banded with lower spires (Figs 5 and 8D), adult and juvenile *C. apertus* were found to consistently have larger apertures compared with *C. aspersum* specimens, with AH proportionally larger (Fig. 5, Fig. 8B AH:SW, Fig. 8C AH:AW). Relative aperture size is known to affect desiccation resistance in snails (Perrot *et al.,* 2007), and the large-apertured Green Garden Snails burrow freely into soil and seal their shells with a strong white coloured epiphragm (see Fig. 2F) during drought aestivation (Kershaw, 1979; DPI, 2011), while the other three smaller-apertured species *(C. aspersum, T. pisana, E. vermiculata)* generally seal their shells against vegetation and other objects (Baker, 2002; Yildirim, 2004; Stanisic *et al.,* 2010). A further distinguishing feature of living Green Garden Snails is the characteristic noise they often make when disturbed (Schultes, 2012).

Mantle, head and foot morphology

The soft body of adult garden snails generally appears darker than juveniles, and the surface develops rough tubercles with age (see Fig. 2). The colour of the soft body can be variable within species, however, generally, *C. aspersum* are greenish-grey with a pale dorsal stripe (Fig. 2); *C. apertus* are variably light or dark, but are often cream coloured, with dark dorsal stripes (Fig. 2); *E. vermiculata* are reported as being cream with a dark mantle (Stanisic *et al.,* 2010); while the soft body parts of *T. pisana* are pale overall.

Molecular variation

Comparisons of the DNA sequences obtained in the present study with sequences on GenBank (Blastn searches) revealed the most similar COI and ITS2 DNA sequences (< 5% difference COI, and < 3% difference ITS2) to be respectively with *C. aspersum, C. apertus* and *T. pisana*, confirming the morphological species identifications.

Several COI haplotypes were detected in *C. aspersum* and *C. apertus* (< 1.5% sequence divergence within each species), while only a single haplotype was found in the Victorian *T. pisana* (Fig. 9), which differed from the previous

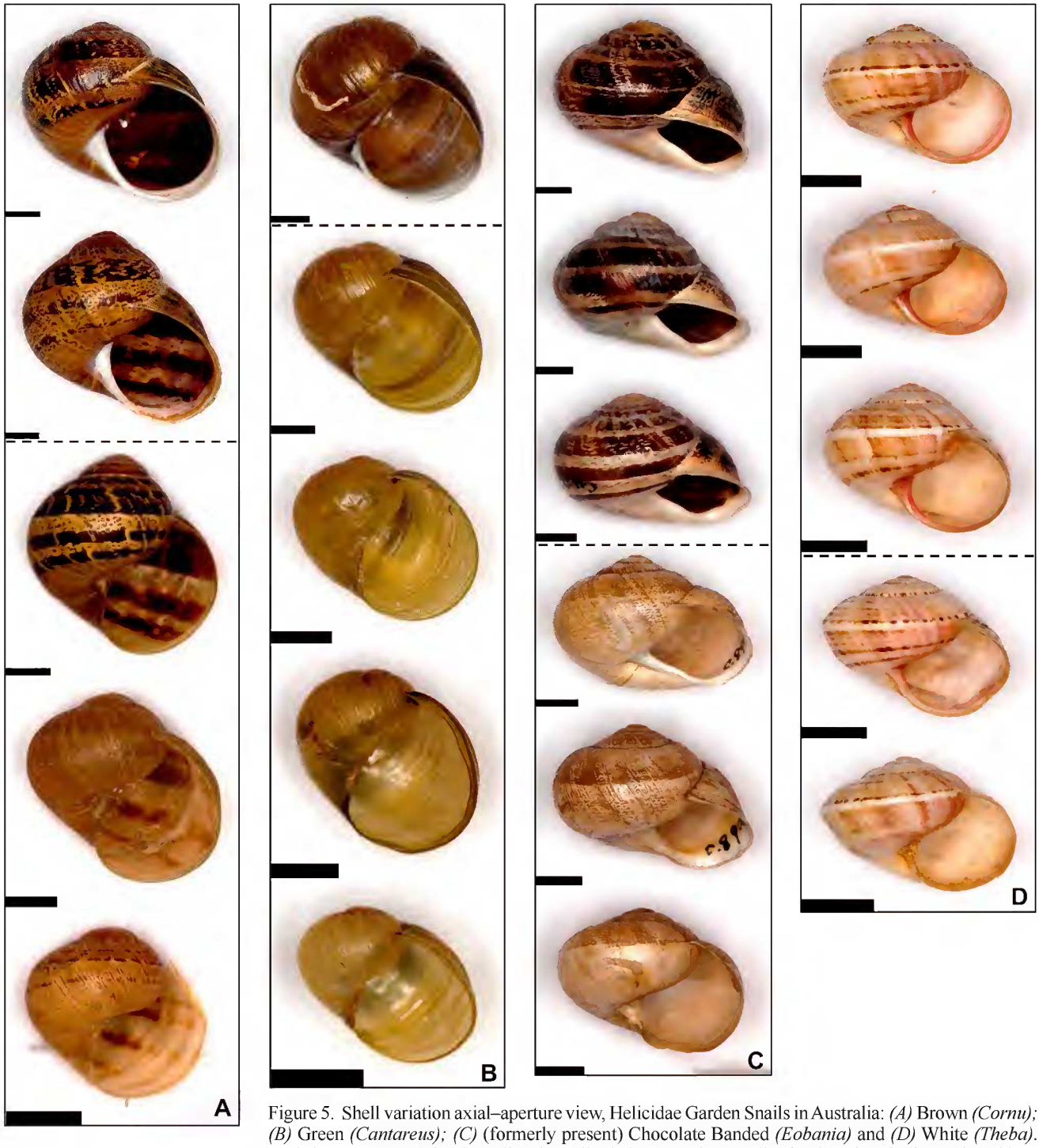

Figure 5. Shell variation axial–aperture view, Helicidae Garden Snails in Australia: *(A)* Brown *(Cornu); (B)* Green *(Cantareus); (C)* (formerly present) Chocolate Banded *(Eobania)* and *(D)* White *(Theba)*. Scale bar = 5 mm; adults above line, juveniles below; specimen collection locations indicated in Table 2.

T. pisana samples examined from Perth, Western Australia (Daumer *et al.*, 2012) by a single base. The ITS2 sequences obtained in the present study contained a small number of heterozygous bases, and were very similar within each species (< 1% divergent). Indels were present in ITS2 in all *C. aspersum*, half of the *C. apertus*, and none of the *T. pisana*; these indels prevented some small sections of the ITS2 sequences from being fully determined.

The COI DNA sequences obtained here for the "Universal" DNA barcoding region for *C. apertus* are the first to be obtained for this species and submitted to BOLD. Currently there is also a single unpublished sequence for this species on GenBank (JX911286, from an unspecified collection locality) which is > 4% divergent from the sequences obtained in the present study (Fig. 9). Multiple COI sequences are available on GenBank for Brown Garden Snails (e.g., Grande *et al.*, 2004; Greve *et al.*, 2010; Neiber *et al.*, 2011; Régnier *et al.*, 2011; Rada *et al.*, 2012), which occur in three distinct genetic groups, with all Australian Brown Garden Snail specimens examined being closely related to each other, matching closely with specimens previously examined from western Europe (Fig. 9). The four Helicidae species examined here, including additional sequences available on GenBank (i.e. previously examined *C. aspersum, C. apertus, E. vermiculata* and *T. pisana*), differ from each other by an average of more than 15% (Table 5, Fig. 9).

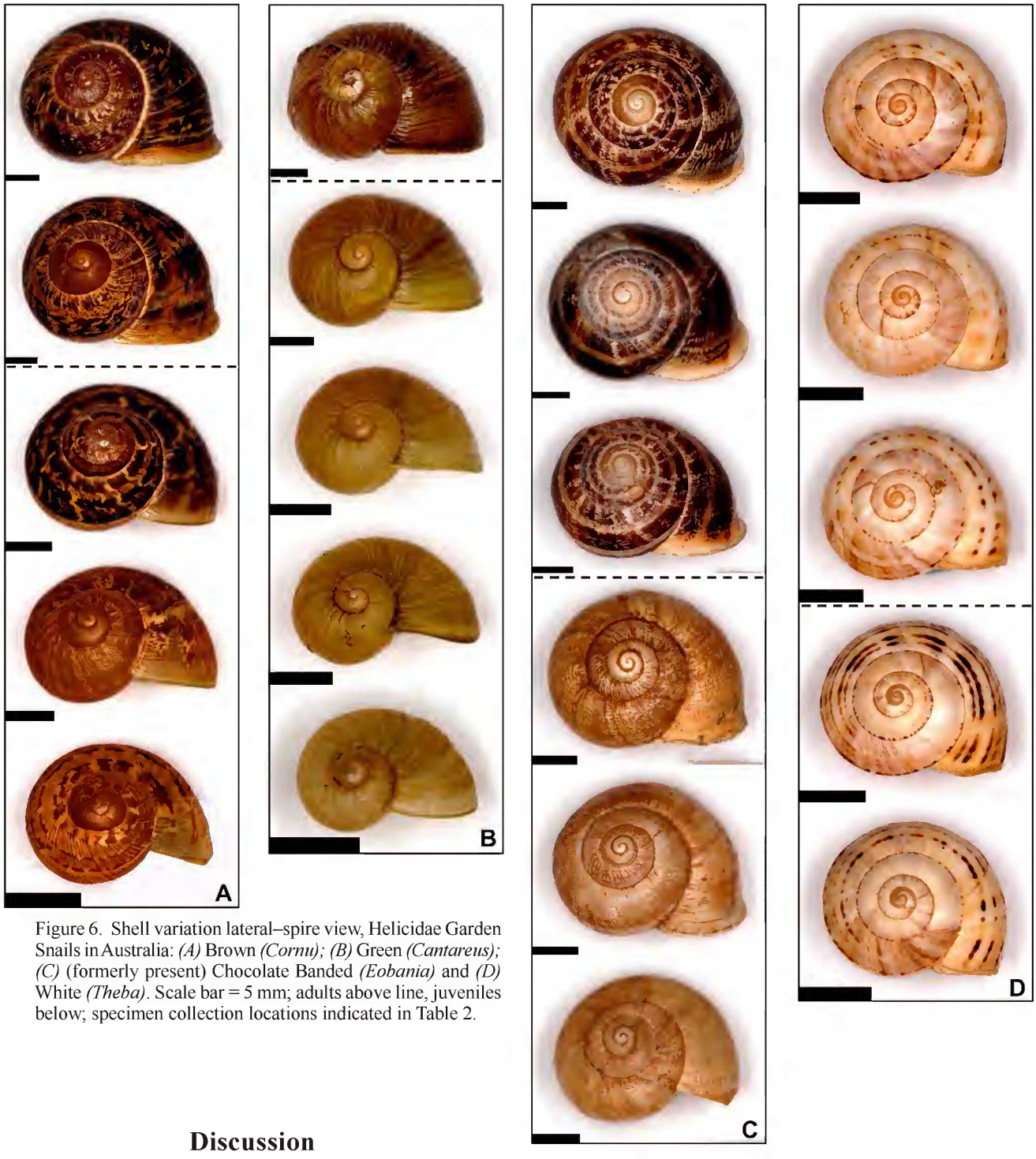

Figure 6. Shell variation lateral–spire view, Helicidae Garden Snails in Australia: *(A)* Brown *(Cornu); (B)* Green *(Cantareus); (C)* (formerly present) Chocolate Banded *(Eobania)* and *(D)* White *(Theba)*. Scale bar = 5 mm; adults above line, juveniles below; specimen collection locations indicated in Table 2.

Discussion

Identification of Helicidae in Australia

Many higher level groups of mollusc are yet to be fully phylogenetically resolved, however the family Helicidae is a well-supported group both morphologically and genetically (Wade *et al.*, 2008; Razkin *et al.*, 2015). Examinations of literature and collection reference specimen records (present study) show that only four species (in four genera) of Helicidae (approximately 1% of the world fauna) appear to have managed to establish in Australia to date—with one of these now locally extinct—despite regular border interceptions of a number of other helicid species (e.g., Table 3).

The four helicid species (above) belong to different genera (however, see Systematics section), and all differ considerably both morphologically and genetically (as demonstrated by the present study). Currently, there are a large number of reference DNA barcode sequences available, with more than 160 Helicidae species (i.e. approximately 40% of species worldwide) already represented on the BOLD database (accessed March 2015). The large genetic differences observed between species make a DNA barcoding approach an extremely valuable tool for confirming the identity of introduced helicid species, as there are a large number of morphologically similar potential

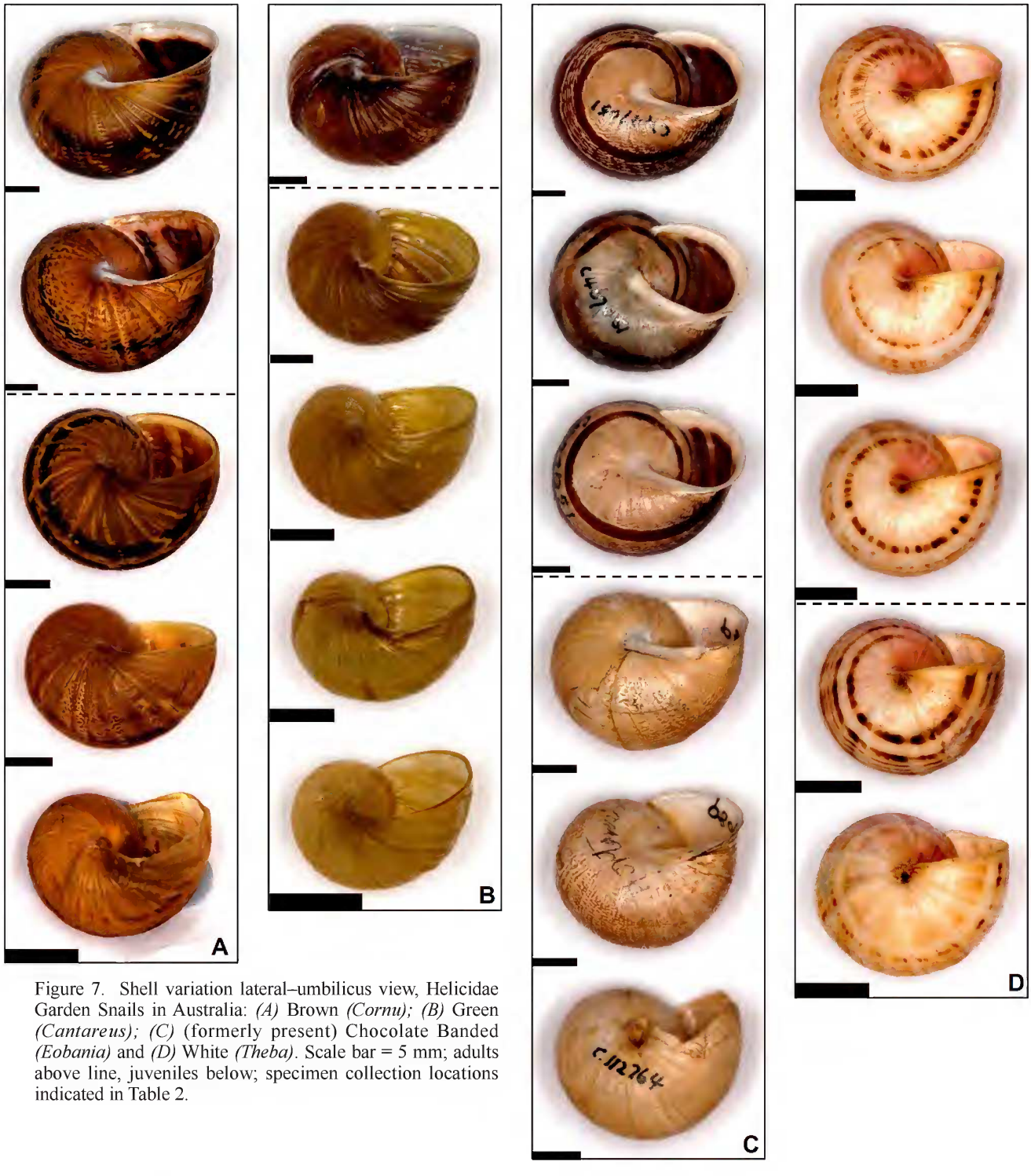

Figure 7. Shell variation lateral–umbilicus view, Helicidae Garden Snails in Australia: *(A)* Brown *(Cornu); (B)* Green *(Cantareus); (C)* (formerly present) Chocolate Banded *(Eobania)* and *(D)* White *(Theba)*. Scale bar = 5 mm; adults above line, juveniles below; specimen collection locations indicated in Table 2.

exotic species of Helicidae worldwide (Schileyko, 2006), some only recently described (e.g., Mumladze *et al.*, 2008), with most morphological keys usually only dealing with specific geographic regions (e.g., Yildirim, 2004; present study). Fortunately, members of additional helicid genera, including those intercepted in recent years—*Arianta, Cepaea, Helix, Marmorana* and *Otala* (L. Watson, pers. comm.)—also appear genetically distinct (Fig. 9).

Genetic variation in Australia

Although sampling was relatively limited in the present study, it does provide an initial indication of potential source populations for Brown and White Garden Snails. Multiple lineages of Brown Garden Snails are known to have spread across the world (Madec *et al.*, 2003; Guiller & Madec, 2010; Galtán-Espitia *et al.*, 2013). In Australia Brown Garden Snails are believed to have been introduced from northern Europe (Smith, 1992), in the present study all Australian *Cornu aspersum* specimens appeared closely related to each other, and were most similar to those previously tested from western Europe (Fig. 9).

Table 3. Recent Australian border interceptions of Helicidae species. (Sourced from OSP Bulletins: June 2011–June 2012, L. Watson pers. comm.).

species	date	state	source	from
Eobania vermiculata	2011-Oct	SA	external container	China
Cantareus apertus	2011-Oct	WA	old Ford utility	USA
Cepaea nemoralis	2011-Dec	Qld	external container	Germany
Eobania vermiculata	2011-Dec	WA	steel formwork	Ireland
Eobania vermiculata	2012-Jan	Qld	external container	Spain
Cepaea nemoralis	2012-Jan	Qld	external container	China
Eobania vermiculata	2012-Mar	SA	container	Unknown
Eobania vermiculata	2012-Jun	Qld	used machinery	Italy
Arianta arbustorum	2012-Jun	Qld	railway wagons	China

Table 4. *(A)* Shell size measurements (ranges, mm) and *(B)* Statistical tests, Discriminant Function Analyses (DA) and Principal Component Analyses (PCA), for four species of Helicidae Garden Snails. Factor 1 accounts for more than 90% of the observed variation. Measurement abbreviations: *AW*, aperture width; *BW*, body-whorl height; *SH*, shell height; *SW*, shell width; and *W*, height of whorls (see Fig. 3).

	SW	AW	SH	BW	AH	W
A						
Brown *(Cornu)*	9.6–31.1	6.0–20.5	8.4–30.6	8.1–27.4	7.1–20.6	0.3–3.2
Green *(Cantareus)*	10.2–25.9	6.7–16.1	9.4–25.1	9.0–23.9	8.3–19.6	0.1–1.6
Chocolate Banded *(Eobania)*	22.1–30.8	12.8–19.4	15.9–24.3	13.8–20.4	11.8–16.7	2.0–4.3
White *(Theba)*	7.7–20.6	4.7–12.9	5.4–15.6	4.8–13.6	4.0–10.8	0.4–2.2
B						
Variable Multicolinearity–Tolerance Statistic (DA)	0.02	0.01	0.01	0.01	0.05	0.00
Correlation between each variable with Factor 1 (DA)	0.5	0.4	0.1	-0.1	-0.1	0.7
Contribution (%) of each variable to Factor 1 (PCA)	18.0	18.5	18.7	17.2	16.5	11.1

Table 5. Average DNA differences (COI locus) within (grey-shaded) and between Helicidae Garden Snail species.

	within sp.	Brown	Green	Chocolate	White
Brown *(Cornu)*	2.6	—	—	—	—
Green *(Cantareus)*	1.5	15.5	—	—	—
Chocolate Banded *(Eobania)*	2.0	16.2	18.4	—	—
White *(Theba)*	5.5	17.6	18.2	18.5	—

White Garden Snails *(Theba pisana)* were first detected in Western Australia in the 1890's (Johnson, 1988), before spreading east, reaching South Australia by 1928 (Pomeroy & Laws, 1967), and the eastern states by the 1990's (Baker, 1986, 2002; Sanderson & Sirgel, 2002). Our study confirms that eastern Australian specimens belong to the subspecies, *Theba pisana pisana*, with the COI haplotype found here being extremely similar genetically (though not identical, see Fig. 9) to a haplotype detected in specimens previously examined from Western Australia, South Africa and the Netherlands (Daumer *et al.,* 2012). Future genetic assessments of additional populations across Australia would be valuable in understanding the introduction history of this species in Australia.

Detailed studies screening population molecular variation in all Helicidae species in Australia would be valuable in assessing the introduction history and possible original source populations of each species. Genetic assessments for the other two species (Green and Chocolate Banded Snails) were not possible in the present study. Australian Green Garden Snails are believed to have been originally introduced from southern Europe (Smith, 1992), however possible source populations of the eastern Australian specimens (either internationally or from within Australia) are yet to be examined. Possible source populations of Chocolate Banded Snails are beginning to be examined worldwide with a number of *Eobania vermiculata* subspecies recognised (e.g., Rada *et al.,* 2012), however, *E. vermiculata* was only available as dry shells in the present study.

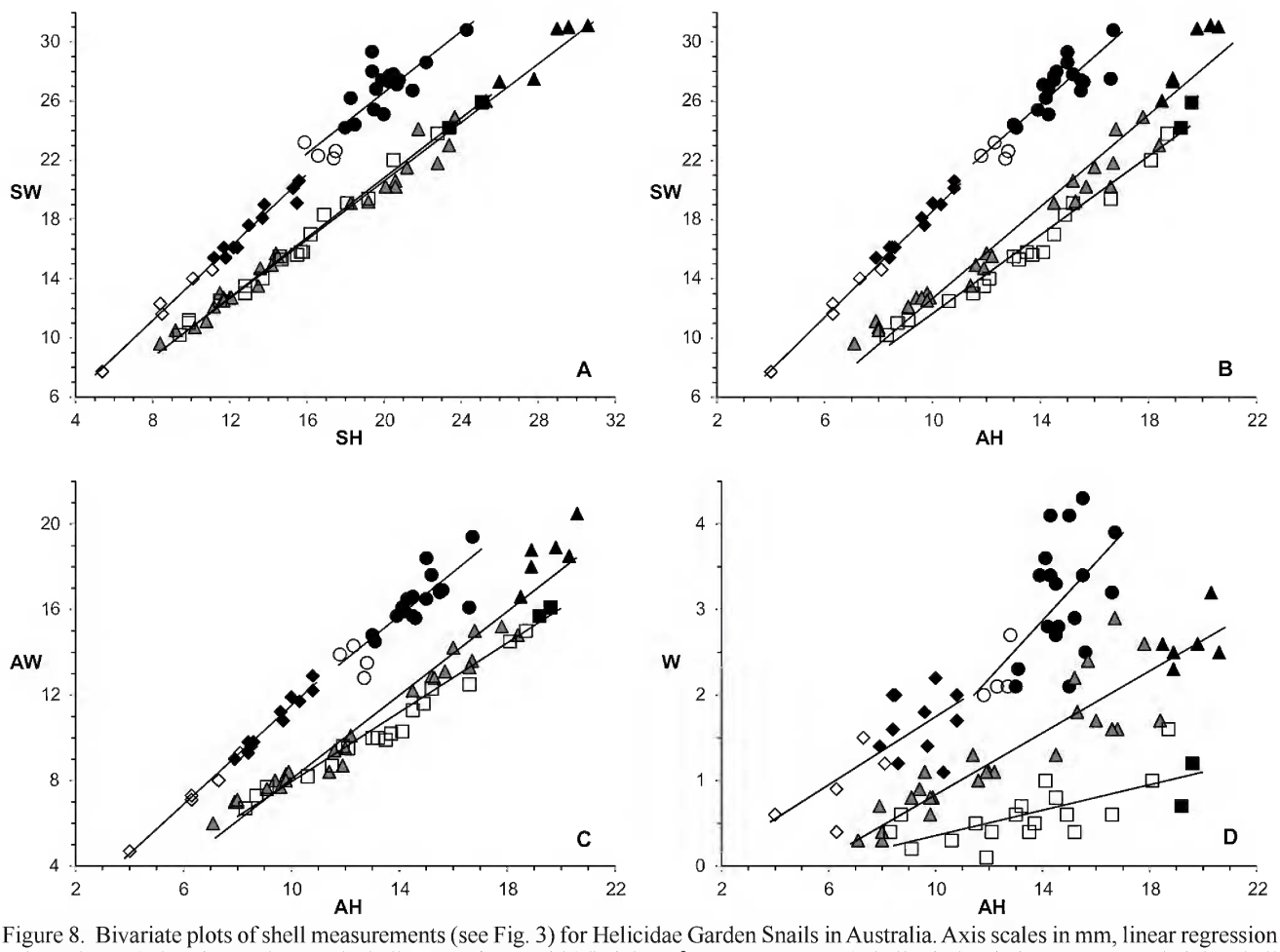

Figure 8. Bivariate plots of shell measurements (see Fig. 3) for Helicidae Garden Snails in Australia. Axis scales in mm, linear regression values shown below in brackets. *(A)* Shell proportions: width/height ($R^2 = 0.73–0.99$); *(B)* shell width relative to aperture height: shell width/aperture height ($R^2 = 0.77–0.99$); *(C)* aperture proportions: width/height ($R^2 = 0.71–0.99$); *(D)* development of whorls: height of whorls/aperture height ($R^2 = 0.36–0.85$). Symbols: ▲ *Cornu;* ■ *Cantareus;* ● *Eobania* and ◆ *Theba*. Adults: black filled shapes; juveniles: unfilled symbols *(Cantareus, Eobania, Theba)* or grey *(Cornu)*.

Green Garden Snails in eastern Australia

Generally, dispersal of garden snails is known to occur both (relatively slowly) naturally from adjacent areas and (relatively quickly) through colonisation to new areas accidentally on human transported goods (e.g., Hely *et al.,* 1982; Barker & Watts, 2002; Peltanová *et al.,* 2012). Human mediated transport is no doubt responsible for Green Garden Snails becoming established in eastern Australia. The first detection occurred on a property near Cobram in north-eastern Victoria in September 2011 (specimens listed in Systematics section). As part of Biosecurity DEDJTR Victoria's surveillance and eradication activity, regular field surveys were conducted until December 2011 on the original property as well as adjoining properties. Currently, there is still restricted movement of plant material and limited surveillance for Green Garden Snails being carried out in Victoria (G. Berg, pers. comm.).

Earlier historical records of Green Garden Snails from Hobart, Tasmania, cited by Kershaw (1991) appear to be an error, as the original record in Petterd & Hedley (1909) refers to *Geostilbia aperta* (Swainson) which is not a member of Helicidae (family Ferussaciidae), rather than *Helix aperta* (synonym for Green Garden Snails). The ferrussaciid referred to in Petterd & Hedley (1909) was probably *Cecilioides acicula*, a species which still occurs near Hobart (M. Shea, pers. obs.).

Conclusions

Introduced Helicidae garden snails in Australia are associated with human modified habitats and can be serious pests, being generalist feeders and capable of breeding up to large numbers. Our study has shown that a relatively small number of species (n = 3) are currently established in Australia, however future incursions of other exotic helicids are very likely. We provide a baseline which should assist in the early detection of any additional newly introduced Helicidae species into Australia. We have also documented the first occurrence of Green Garden Snails in eastern Australia where they may become a significant agricultural pest. Accurate species identification of all lifestages is essential for biosecurity (e.g., Blacket *et al.,* 2012, 2015) and in the present study introduced Helicidae snail adults were found to be morphologically distinctive for a number of diagnostic size, colour and pattern differences in shell and body morphology, however the juveniles can be problematic. The large degree of molecular differentiation observed between these species could be used to identify suspect-exotic specimens in a DNA barcoding approach, and should prove especially valuable for identification of small juvenile specimens.

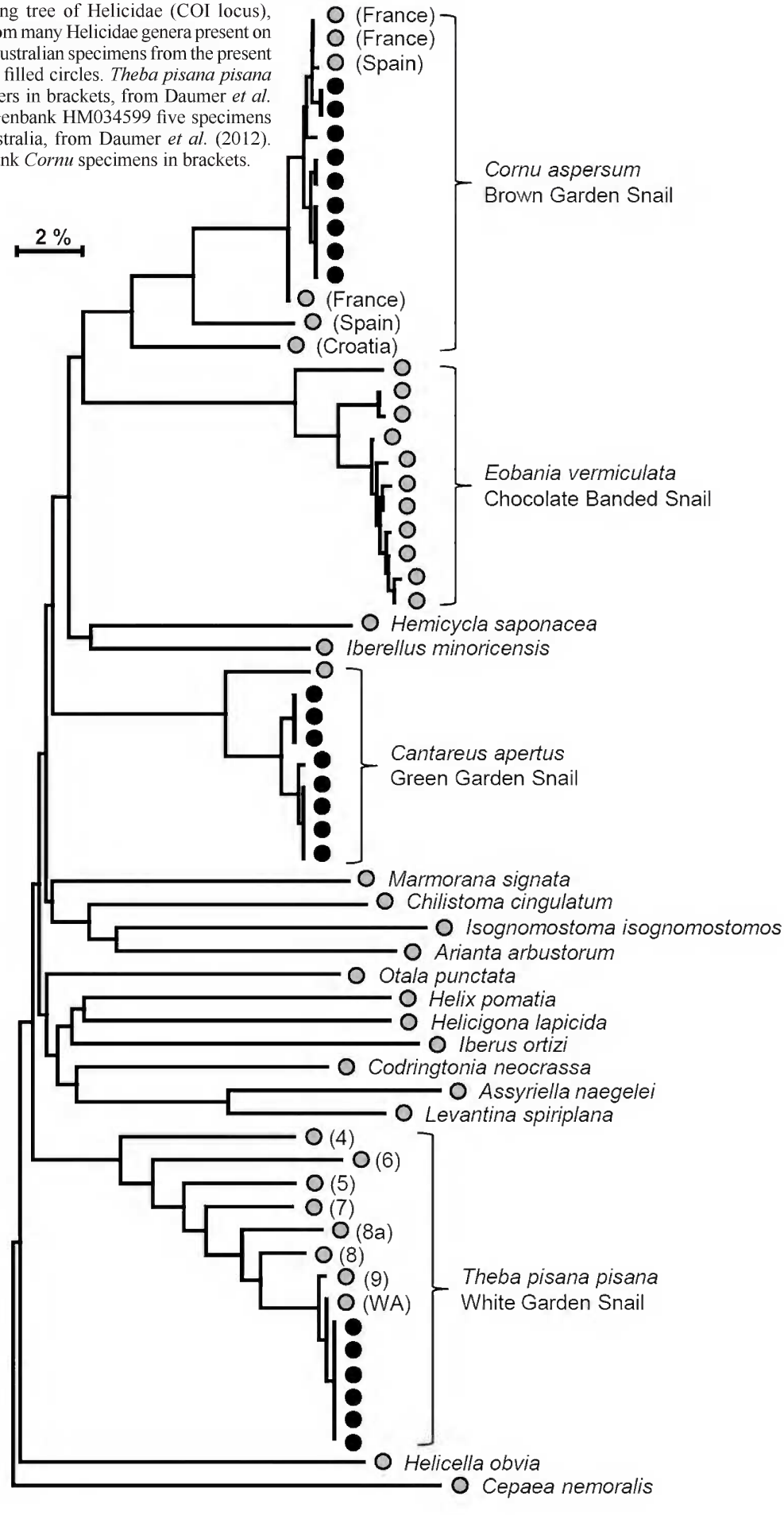

Figure 9. Neighbour-joining tree of Helicidae (COI locus), including DNA sequences from many Helicidae genera present on GenBank. Sequences from Australian specimens from the present study are indicated as black filled circles. *Theba pisana pisana* lineages indicated by numbers in brackets, from Daumer *et al.* (2012), *T. pisana* (WA) = Genbank HM034599 five specimens collected from Western Australia, from Daumer *et al.* (2012). Country of origin for Genbank *Cornu* specimens in brackets.

Systematics

GASTROPODA

STYLOMMATOPHORA

Family HELICIDAE Rafinesque, 1815

Genus *Cornu* Born, 1788

Cornu aspersum (Müller, 1774)

Objective synonyms—*Helix aspersa, Cantareus aspersa, Cryptomphalus aspersus.*

Common names—Brown Garden Snail, Common Garden Snail.

Material examined. *New South Wales:* AM C121130 Jenolan Caves; AM C430603 St. Peters, Sydney; AM C430605 Seal Rocks headland south of Forster; AM C430607 Timor Caves camping ground, Upper Hunter River; AM C456977 east of Glen Davis at end of Capertee River track; AM C430602 Swansea, south of Newcastle. *Victoria:* Blackburn South VAITC3037, VAIC ×1; Cobram VAITC3072, 3073; Doncaster VAITC3022, VAIC ×3; Knoxfield VAITC3015, 3071, VAIC ×7; Irymple VAITC3077, 3078, 3080, VAIC ×11; Preston VAITC3076, VAIC ×4; Ringwood East VAITC3047, 3048, VAIC ×5.

Diagnosis. Shell: large (up to 4 cm), thin, raised spire, globose, 4–5 whorls increasing rapidly with body-whorl greatly flared, aperture large and rounded, umbilicus closed, shell lip thickened white and strongly reflected out in adults, rounded whorls in both adults and juveniles. Shell colour: light brown with darker spiral bands and yellow flecks. During aestivation: possesses either thin and clear or thickened and greyish green epiphragm, that is not convex positioned inside the aperture. Soft body: greenish-grey with a pale dorsal stripe. Adults: identified by the presence of a thickened reflected lip, indicating maturity and the cessation of growth (*sensu* Madec *et al.,* 2003).

Distribution in Australia. Present in parts of all Australian states and territories.

Remarks. The species identification of VAITC specimens (above) was confirmed in the present study through DNA barcoding (ITS2 & COI) and comparison with validated AM specimens. In this study we employed the generic name *Cornu,* as is currently accepted (ICZN Case 3518—ICZN, 2015). Phylogenetic estimates of relationships within Helicidae show that Green and Brown Garden snails are closely related based on both 16S (Manganelli *et al.,* 2005; Groenenberg *et al.,* 2011; Razkin *et al*., 2015) and ITS2 (Wade *et al.,* 2006; Régnier *et al.,* 2011; Razkin *et al*., 2015) DNA sequences, with most of these previous studies placing these two species in the same genus, *Cantareus,* as proposed by Guisti *et al.* (1996), on the basis of a shared morphological internal synapomorphy. However, the majority of recent studies place Brown Garden Snails in *Cornu* (as in the present study). Indeed, in the present study Brown and Green Garden Snails do appear to be substantially genetically distinct based on COI variation (Table 5, Fig. 9), adding some support to the placement of these species in different genera. The placement of Brown Garden Snails in the genus *Helix* is not supported by molecular evidence, with species of *Helix* now placed in a different tribe from *C. aspersum* and *C. apertus* (e.g., Razkin *et al*., 2015). *Cornu* currently contains a single species (CABI species datasheet, http://www.cabi.org/isc/datasheet/26821).

Genus *Cantareus* Risso, 1826

Cantareus apertus (Born, 1778)

Objective synonym—*Helix aperta.*

Common names—Green Garden Snail, Green Snail, Singing Snail.

Material examined. *Victoria:* Cobram VAITC3032–3036, 3103, 3104, VAIC ×10; Cobram South VAITC3014, 3018, 3019, 3020, 3021, 3066–3070, VAIC ×9. *Western Australia:* AM C135546 Wanneroo, north of Perth; VAIC Neerabup ×2.

Diagnosis. Shell: medium (up to 3 cm), very thin, low spire, globose, 3–4 whorls increasing rapidly with body-whorl greatly flared, aperture extremely large and rounded, umbilicus closed, shell lip thickened white internally only and not reflected out in adults, rounded whorls in both adults and juveniles. Shell colour: olive green to brown with no banding pattern. During aestivation: possesses convex white thick epiphragm, extending from edge of aperture. Live animal: can make a distinctive noise when disturbed. Soft body: usually cream to dark-grey coloured, with dark dorsal stripes. Adults: identified by size and degree of thickening of the shell-lip.

Distribution in Australia. Present in WA at Wanneroo, Neerabup NP, Herne Hill and Bayswater, Perth; and in north-eastern Victoria, near Cobram.

Remarks. The species identification of VAITC specimens (above) was confirmed in the present study through DNA barcoding (ITS2 & COI) and comparison with validated AM specimens. Formerly regarded as belonging to *Helix*, it is now considered to be in the genus *Cantareus* (Schileyko, 2006), see ICZN comments above under *Cornu*. *Cantareus* currently contains a single species (Schileyko, 2006).

Genus *Eobania* Hesse, 1913

Eobania vermiculata (Müller, 1774)

Objective synonym—*Helix vermiculata.*

Common names—Chocolate Banded Snail, Chocolate-band Snail.

Material examined. New South Wales: Sydney, Bronte, Waverley Cemetery AM C407051 ×18 (adults); Sydney, Ryde AM C089089 ×4 (juveniles); Sydney, Ryde AM C112764 ×4 (juveniles with damaged lips).

Diagnosis. Shell: medium (up to 3 cm), thick to thin, short and wide, raised spire, subglobose, 5 whorls, body-whorl moderately flared, aperture small and compressed, umbilicus closed, shell lip thickened white and strongly reflected out in adults, rounded whorls in both adults and juveniles. Shell colour: light brown to yellow usually with continuous thick dark brown and white spiral bands and yellow flecks. Soft body: cream with a dark mantle (Stanisic *et al*., 2010). Adults: identified by strongly reflected lip and a well-developed columellar plait covering the umbilicus (Stanisic *et al*., 2010).

Distribution in Australia. Locally extinct, formerly present in NSW, near Sydney, and Leven River, Ulverstone Tasmania, but no longer occurs at either location.

Remarks. Validated AM specimens were used for species identification in the present study. *Eobania* currently contains a single species (Schileyko, 2006), with several subspecies present in the natural range of *E. vermiculata* (e.g., Rada *et al.,* 2012). The sub-specific designation of the Australian specimens is not yet determined.

Genus *Theba* Risso, 1826

Theba pisana (Müller, 1774)

Objective synonym—*Helix pisana.*
Common names—White Garden Snail, White Gardensnail, White Italian Snail.

Material examined. *New South Wales:* AM C088901 Nelson Bay, Port Stephens; AM C206180 Redhead Beach, Newcastle; AM C168582 Avalon Beach, Sydney; AM C407047 Waniora Point, Bulli; AM C348456 Cunjurong Point, E of Lake Conjola; AM C404152 Cudmirrah Beach near Sussex Inlet; AM C334544 Aslings Beach, Twofold Bay, Eden. *Victoria:* Venus Bay VAITC3831–3837, VAIC ×10; Port Campbell VAIC ×3, 3 km North Port Campbell VAIC ×3.

Diagnosis. Shell: small (up to 2 cm), thick to thin, tall and narrow, raised spire, subglobose, 5 whorls, body-whorl moderately flared, aperture medium and rounded, narrow umbilicus, shell lip thickened pink internally only and not reflected out in adults, whorls rounded in adults angulate in juveniles. Shell colour: white usually with broken thin dark brown spiral bands and chevrons. Soft body: pale overall. Adults: identified by the presence of rounded body-whorls (Stanisic *et al.,* 2010).

Distribution in Australia. Present in southern Australia: NSW, Tas., Vic., SA, WA.

Remarks. The species identification of VAITC specimens (above) was confirmed in the present study through DNA barcoding (ITS2 and COI) and comparison with validated AM specimens. The genus *Theba* contains at least 18 species in its natural range (Greve *et al.,* 2010), but only *Theba pisana pisana* occurs worldwide (e.g., Cowie *et al.,* 2009; Greve *et al.,* 2010; Däumer *et al.,* 2012). Australian specimens belong to *T. p. pisana* (Däumer *et al.,* 2012; current study). CABI species datasheet: http://www.cabi.org/isc/datasheet/62094.

AUTHOR CONTRIBUTION STATEMENT. All authors were involved in the conception and design of this research, identifying diagnostic specimens during the initial eastern Australian detection of Green Garden Snails, and contributing to the systematics section. MB conducted the morphometric and molecular experimental work, and produced the specimen images (apart from Figs 2 and 4). MS provided expert support through validating identifications of reference specimens, providing unpublished background information and assisting with the morphometrics section. All authors collected snails for this study and contributed to the writing of the manuscript.

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